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Characterization of the Lysogenic Repressor (*c*) Gene of the *Pseudomonas aeruginosa* Transposable Bacteriophage D3112

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Bacteriophage D3112 is a Mu-like temperate transposable phage of *Pseudomonas aeruginosa*. Genetic mapping and DNA sequence analysis have identified the left end of the phage genome as encoding the transposase enzyme (*A*) and the lysogenic (*c*) repressor. The *c* open reading frame (ORF), located at the leftmost end of the phage genome and transcribed from right to left, has four possible GTG initiation codons. Using site-directed mutagenesis, each of the four GTG codons was modified to GTA, which cannot serve as an initiation codon. Plasmids were constructed expressing either the wild-type repressor ORF or the ORFs containing the mutated GTA codons. When introduced into *Pseudomonas aeruginosa,* no immunity to superinfection by D3112 was observed when the second GTG had been mutated. Northern blotting analysis demonstrated that the D3112 *c* repressor is transcribed as a 900-nt mRNA. The promoter region was defined by transcriptional *lacZ* fusions and primer extension analyses to bp 972–940 from the left end of the phage genome. When the D3112 c repressor was overexpressed and purified as a fusion protein with a C-terminal six-histidine extension (cts15-His6), it showed high affinity for a 261-bp *Pvu*II fragment localized directly upstream of the *c* repressor ORF. Our results indicate that although D3112 c shows higher amino acid similarity to the λ family of repressors than it does to those of Mu and D108, it appears that its structure and function more accurately reflect an evolutionary ancestry with those from transposable coliphages Mu and D108.

INTRODUCTION

Bacteria of the genus *Pseudomonas* are infected by more than 60 distinct temperate, transposable bacteriophages, including phage D3112 (Akhverdian *et al.,* 1984; DuBow, 1994). Such transposable bacteriophages are rare in the Enterobacteriaciae. The two known transposable coliphages, Mu and D108, have been well studied and are able to function as both transposons and as viruses (reviewed in Pato, 1989; DuBow, 1994). In the lytic cycle, the infecting phage DNA first integrates conservatively (via DNA transposition) into the bacterial chromosome at random locations. These prophages then amplify their genomes through replicative transposition to new locations within the host chromosome. In the lysogenic state, the prophage lytic functions are repressed by the action of a repressor (the *c* gene product).

Bacteriophage D3112 has a 38-kbp linear double-stranded DNA genome and possesses a genetic organization similar to that of the transposable coliphages Mu and D108 (Krylov *et al.,* 1980a,b; Yanenko *et al.,* 1983, 1988; DuBow, 1994). Genetic mapping, using deletion mutants of an RP4::D3112 plasmid (Krylov *et al.,* 1982; Yanenko *et al.,* 1983, 1988; Gerasimov *et al.,* 1985), was used to localize the lytic-lysogenic regulatory region to the leftmost end of the phage genome. Bacteriophage D3112, like the two coliphages, is also capable of acting as an insertional mutagen in Pseudomonads, and lysogens of the phage have insertions in many different locations (Plotnikova *et al.,* 1983; Rhemat and Shapiro, 1983).

Transposable phages have proven to be enormously useful in the study of gene structure and expression in their hosts. Mini-D3112 transposons, which lack the genes essential for phage growth but retain the terminal sequences required *in cis* for transposition, have been developed for use in *Pseudomonas* strains (Darzins and Casadaban, 1989a,b). An understanding of the lytic-lysogenic switch and regulation of phage-mediated DNA transposition would be beneficial for both the future development of D3112 as a genetic tool and our understanding of gene regulation in *P. aeruginosa* and its bacteriophages.

The leftmost 5.5 kbp of the D3112 genome has been cloned and sequenced (Autexier *et al.,* 1991; Ulycznyj *et al.,* 1995). Several open reading frames (ORFs) have been identified, and their locations suggest strong functional similarity with the leftmost ends of Mu and D108 (Yanenko *et al.,* 1983, 1988; DuBow, 1994). The putative lysogenic repressor, encoded by the *c* gene, is tran-

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TABLE 1

Immunity to D3112 Superinfection Conferred by Cloned D3112 Left-End DNA-Containing Plasmids in PAO1

	Titer of phage lysate (pfu/ml)									
Plasmid	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}
32°C										
None	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
pTJS140	−	−	−	+	++	++	+++	+++	+++	+++
pSWL12	−	−	−	−	−	−	−	−	−	−
pKAS305	−	−	−	−	−	−	−	−	−	−
pKAS306	−	−	−	++	++	+++	+++	+++	+++	+++
pKAS307	−	−	−	−	−	−	−	−	−	−
pKAS308	−	−	−	−	−	−	−	−	−	−
pOF6	−	−	−	−	−	−	−	−	−	−
42°C										
pSWL12	−	−	−	−	+	++	+++	+++	+++	+++
pOF6	−	−	−	−	−	−	−	−	−	−

−, No plaque; +, slight clearing; ++, turbid region of lysis; +++, complete lysis.

scribed from right to left (as in Mu and D108), and a cloned fragment containing the *c* ORF (bp 1174–0) conferred immunity to D3112 superinfection *in vivo* (Autexier *et al.,* 1991). The proteins required for replicative transposition by D3112 (expressed from the *A* and *B* genes) have been identified and characterized (Ulycznyj *et al.,* 1995), whereas the *cip* (control of interaction of phages) function has been mapped to the right of the *c* gene (Gerasimov *et al.,* 1985; Bidnenko *et al.,* 1996). Although located in a position analogous to *ner*, the *cip* function may be more analogous to Rex of λ (reviewed in Court and Oppenheim, 1983; Snyder and Kaufman, 1994), because in lysogens containing multiple D3112 prophages, *cip* was reported to prevent infection by transposable phage B39 (Gerasimov *et al.,* 1985; Bidnenko *et al.,* 1996).

To understand the genetic control of the lytic-lysogenic switch in D3112, the expression and activity of the c repressor gene must be determined. This gene has been mapped to the leftmost end of the phage genome and shown to consist of a single large ORF transcribed from right to left, as in Mu and D108 (Autexier *et al.,* 1991). In this study, we defined the P*c* promoter, localized the repressor start codon, and identified amino acids important to the proper functioning of the repressor. We also purified the D3112 repressor and showed that it binds to a region directly upstream of the D3112 *c* ORF.

RESULTS

Site-specific mutagenesis to determine the c repressor start codon

The original sequencing of the D3112 *c* gene revealed no ATG start codon. Instead, four in-frame GTG codons were found (Autexier *et al.,* 1991). To determine which of the four GTG codons is used as the start codon for translation of the c repressor, plasmid $pB28_L$, containing the leftmost 1.8 kbp of the D3112*c*ts15 genome (Autexier *et al.,* 1991), was used as the target for site-specific mutagenesis. Each of the four putative GTG codons was mutagenized to a GTA codon that also encodes a valine but is no longer able to function as a start codon (Neidhart *et al.,* 1990). The *Nru*I–*Eco*RI fragments (bp 1174–1 of the D3112 *c*ts15 genome) of the mutated plasmids were subcloned into the broad host-range vector pTJS140. *P. aeruginosa* PAO1 cells transformed with the different mutant constructs were tested for their ability to confer immunity to D3112 superinfection (see Materials and Methods) and the results are shown in Table 1. As expected, strains PAO1 and PAL1/pTJS140 were highly susceptible to superinfection *in vivo*. In contrast, strain PAO1/pSWL12 (pTJS140 containing the original left-end fragment and expressing the temperature sensitive repressor *c*ts15) showed a high level of immunity to D3112 superinfection at all phage dilutions when tested at 32°C (Autexier *et al.,* 1991). Among the strains expressing mutant constructs, only PAO1/pKAS206 displayed reduced immunity to D3112 superinfection. Immunity was at a level comparable to that of the vector alone, indicating that the second GTG may serve as the translational start codon.

Cloning and sequencing of the wild-type repressor ORF

Because only the temperature sensitive (ts) repressor had been previously sequenced (Autexier *et al.,* 1991), we sought to determine the sequence of the wild-type D3112 c repressor protein. To accomplish this, the left-end *Hin*dIII fragment from the D3112 c^+ phage genome was cloned into the vector pUC119, generating plasmid

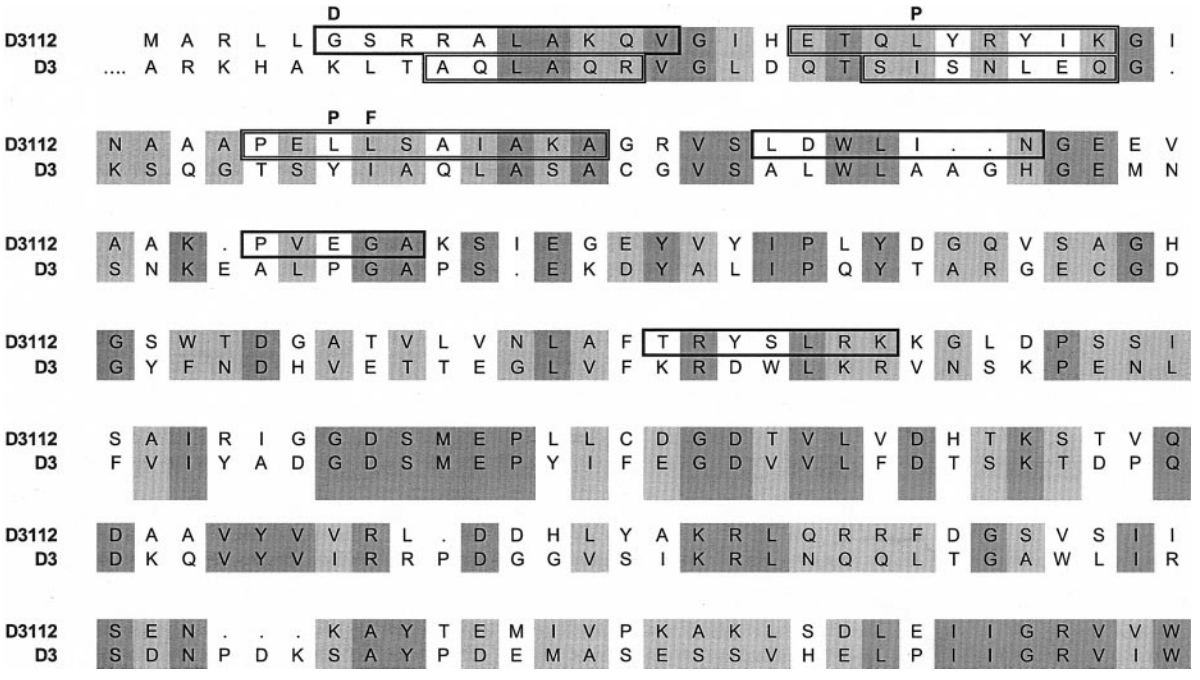

FIG. 1. Comparison of the amino acid sequences of the lysogenic repressor proteins of *Pseudomonas* phages D3112 and D3. Dark gray indicates identity between amino acids, light gray indicates similarity between amino acids. Boxed amino acids indicate predicted helices of D3112 (see text) including the predicted helix-turn-helix motifs of both proteins (double lines). Shown above the D3112 sequence is the location of the *c*ts mutation (D6G) and the three repressor mutants L22P, L36P, and L37F.

pOF4. DNA sequencing revealed a single TA→CG mutation at bp 780 (Autexier *et al.,* 1991). This single-bp mutation results in a ts mutant having the change Gly6Asp (Fig. 1). In addition, although the ts repressor functions only at 32°C (Autexier *et al.,* 1991), plasmid pOF6, containing the wild-type repressor ORF, conferred immunity to D3112 superinfection at 42°C as well as at 32°C (Table 1).

Isolation of repressor mutants unable to confer immunity

To define important motifs of the c repressor protein, plasmid pSWL12 was treated with the chemical mutagen hydroxylamine (HA), which induces either GC→AT or AT→GC transition mutations (Freese *et al.,* 1961a,b; Tessman *et al.,* 1967). These mutated plasmids were electroporated into PAO1 cells, and the resulting strains were screened for loss of immunity to D3112 superinfection using an *in vivo* immunity assay (Levin and DuBow, 1989; see Material and Methods). From the primary screening, 11 plasmids with reduced ability to confer immunity to D3112 superinfection were isolated (data not shown). The *Pst*I–*Eco*RI fragment from these 11 plasmids was cloned into pTJS140 and again screened to confirm that this loss of immunity was associated with a mutation within the *c* ORF and not within the vector itself. After this second round of screening, seven of the original plasmids were still unable to confer immunity to superinfection *in vivo* (Table 2). These seven plasmids were subsequently subcloned into pUC119 for sequencing. Of the seven plasmids, five had an AT→GC mutation at position 732, resulting in the change Leu22Pro (e.g., pOF7, Table 2). One mutant (pOF8) had an AT→GC at position 690, resulting in the change Leu36Pro. The final mutant had a GC→AT change at position 688, resulting in Leu37Phe (pOF14).

Analysis of P*c*, the D3112 c repressor promoter

The promoter for the *c* ORF (P*c*) was located using β-galactosidase assays. Fragments spanning a site from within the *c* ORF to various sites within the upstream region were subcloned into the *lacZ* transcriptional fusion broad-host-range plasmid pDN19lacΩ (Totten and Lory, 1990) (Fig. 2). *P. aeruginosa* PAO1 transformed with pKAS220 (containing an *Nru*I–*Hin*cII fragment from bp

TABLE 2

Identification of Mutations within Plasmids (and in the Protein ORF) that Conferred Immunity after a Second Screening

Plasmid	Mutation[a]	
pOF7	AT → GC	L22P
pOF8	AT → GC	L36P
pOF14	GC → AT	L37F

[a] Mutations are indicated as bp mutation followed by mutation within the coding region of the protein.

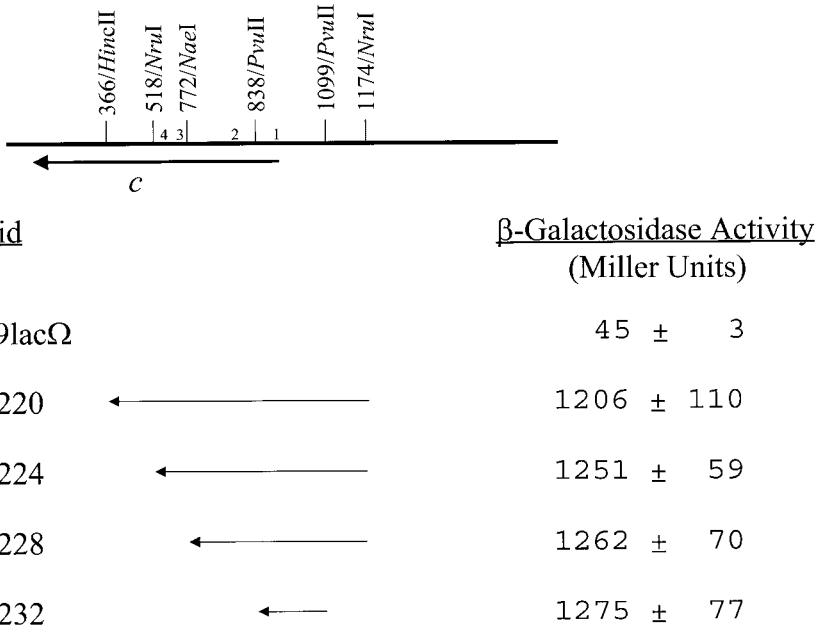

FIG. 2. Analysis of deletion constructs for P*c* promoter activity. The insert in each plasmid, cloned just 5′ of the promoterless β-galactosidase gene in pDN19lacΩ is shown along with levels of β-galactosidase activity in *P. aeruginosa* strain PAO1. The results represent the average of a minimum of three separate experiments, and standard deviations are shown. The arrow reflects the direction of transcription through the promoterless *lacZ* gene. (Top) A restriction enzyme map of the very left end of the D3112 genome with the location of the *c* repressor ORF and positions of the four putative GTG start codons (1→4).

1174–366) produced 1206 Miller units of β-galactosidase. Further deletions of this fragment were cloned into pKAS224 (*Nru*I–*Nar*I), pKAS228 (*Nru*I–*Nae*I), and pKAS232 (*Pvu*II–*Pvu*II). PAO1 strains transformed with these constructs showed no statistically significant changes in promoter activity. A *Pvu*II fragment spanning bp 1099–838 (pKAS232) was the smallest fragment to confer promoter activity.

To determine the size of the *c* transcript, total cellular RNA was isolated from PAO1, PAS429, and PAS429^{IND} (PAS429 induced; see Materials and Methods) cells, subjected to electrophoresis on a 1.2% agarose–formaldehyde gel, and transferred to a nitrocellulose membrane. The *c* gene (bp 770–170) was used as the hybridization probe and identified a 900 nt transcript from PAS429 cells (Fig. 3).

The positions of the P*c* promoter and the transcriptional start site were mapped using primer extension (Fig. 4). The transcriptional start site was localized to position 940, 154 bp upstream of the translation initiation codon (second GTG).

Overexpression and purification of the D3112 *c*ts repressor protein

To further analyze the D3112 repressor, the *c*ts15 ORF, starting with the second GTG, was cloned into the pET29-b expression vector generating pKAS210 and resulting in the addition of six histidine codons to the 3′ end of the gene. PKAS211 was created by cloning, into pET29-b, a truncated version of the *c*ts15 ORF that commenced at the third GTG. pKAS210 and pKAS211 were transformed into BL21(DE3), and expression of cts15-His6 and Δcts15-His6 was induced as outlined in the

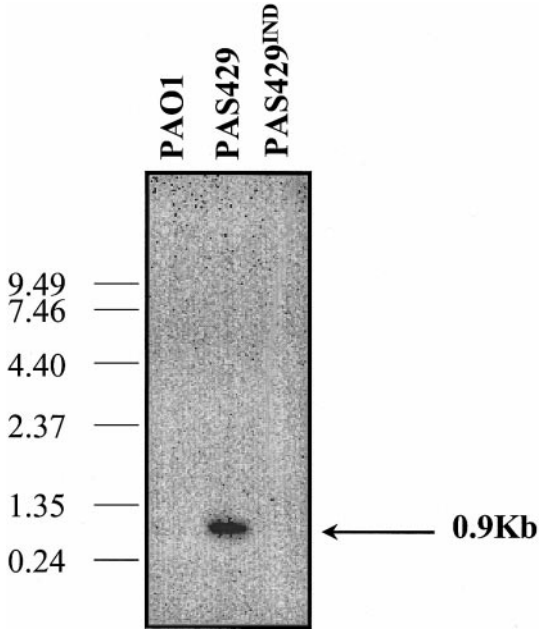

FIG. 3. Northern blot analysis of total cellular RNA from *P. aeruginosa* strains PAO1, PAS429, and PAS429^{IND} using the D3112 *c* gene as a probe. (Left) The 0.4- to 9.5-kb RNA ladder. The arrow points to a 0.9-kb mRNA that hybridized to the probe.

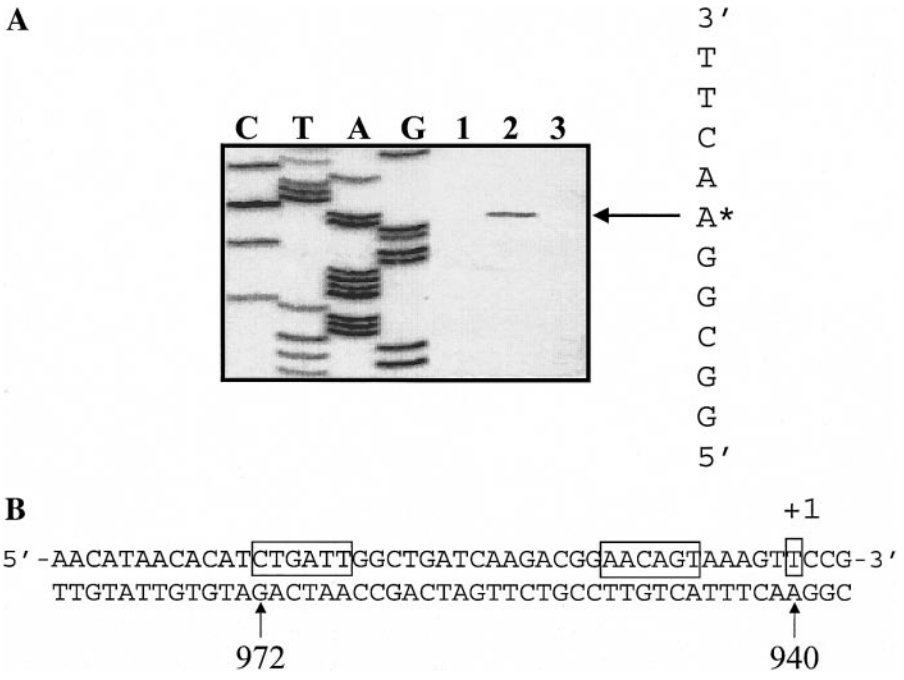

FIG. 4. (A) Transcriptional start site of D3112 *c* mRNA. Lane 1, PAO1; lane 2, PAS429; lane 3, PAS429^{IND}; and lanes C, T, A, and G, nucleotide-specific sequencing reactions. The arrow points to the extended product on the sequence corresponding to the start site. (B) Sequence of the P*c* promoter region (numbered from the left end of the D3112 genome). Boxed nucleotides indicate the putative −10 and −35 regions as well as the +1 transcription start site.

Materials and Methods. Whole-cell extracts from induced transformants were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 5). The cts15-His6 (lane 7) and the Δcts15-His6 (not shown) proteins were purified to greater than 90% homogeneity using Ni-NTA agarose as described in the Material and Methods.

Preliminary characterization of cts-His6 and Δcts15-His6

Band retardation assays (Fried and Crothers, 1981; Garner and Revzin, 1981; Kukolj and DuBow, 1991) were performed to characterize the DNA binding properties of the purified D3112 repressor. A ^{32}P-labeled *Eco*RI–*Hin*dIII fragment from pKAS230, containing the region flanking the P*c*, was incubated with 500 ng of purified cts15-His6. The cts15-His6 protein readily bound to both this specific fragment and to a nonspecific ^{32}P-labeled *Hin*fI fragment from pBR322 (Figs. 6A and 6B, lanes 1, respectively). Upon addition of nonlabeled competitive substrate (2 μg sonicated calf thymus DNA), binding of cts15-His6 to the nonspecific pBR322 fragment was abolished (Fig. 6A, lane 6), whereas the binding of the specific fragment was not completely abolished even after the addition of 5 μg of competitor DNA (Fig. 6B, lane 7). cts15-His6 bound to the specific DNA fragment migrated as two bands (Fig. 6B, lanes 3–7).

Using the same specific and nonspecific ^{32}P-labeled fragments, binding of 500 ng of purified Δcts15-His6 protein was also studied. As illustrated in Figs. 6C (lane 1) and 6D (lane 1), Δcts15-His6 was able to bind weakly to both the nonspecific and specific fragments. Binding by Δcts15-His6 to the nonspecific or specific fragments was abolished upon the addition of only 0.01 or 0.1 μg nonlabeled competitor DNA, respectively (Fig. 6C, lanes 2 and 3).

DISCUSSION

This article describes the initial characterization of the lysogenic c repressor from the *Pseudomonas* transpos-

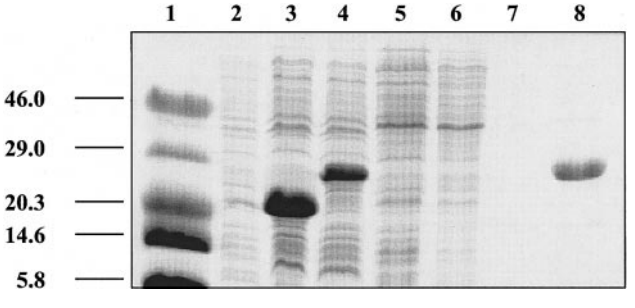

FIG. 5. SDS–PAGE analysis of D3112 c repressors. Lane 1, prestained molecular weight markers; lane 2, crude extract of BL21(DE3)/pET29-b induced for 4 h at 1 mM IPTG; lane 3, crude extract of BL21(DE3)/pKAS211 induced for 4 h at 1 mM IPTG; lane 4, crude extract of BL21(DE3)/pKAS210 induced for 4 h at 1 mM IPTG; and lanes 5–8, some of the purification steps of cts15-His6. Lane 5, flow-through; lane 6, 20 mM imidazole wash; lane 7, 80 mM imidazole wash; and lane 8, elution at 250 mM imidazole.

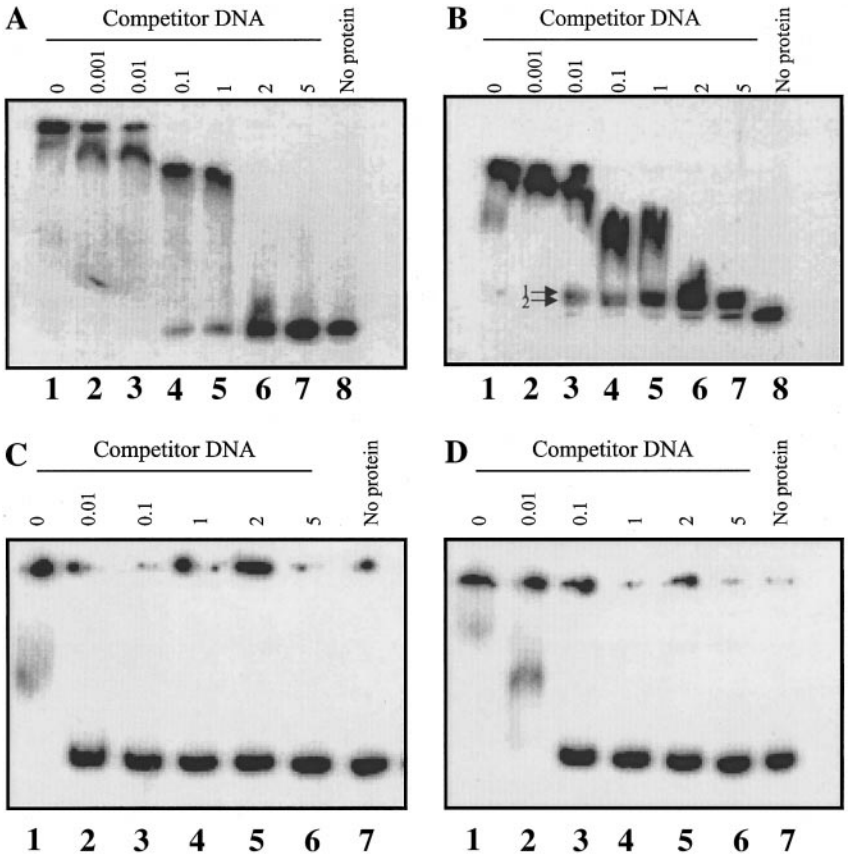

FIG. 6. Band retardation assays with cts15-His6 and Δcts15-His6. (A) Band retardation assay of cts15-His6 with a 154-bp *Hin*fI nonspecific fragment from pBR322. Lanes 1–7, 5000 cpm radiolabeled fragment with 500 ng of purified cts15-His6 incubated with increasing amounts of sonicated calf thymus DNA (competitor DNA). Lane 8, 5000 cpm radiolabeled fragment alone. (B) Band retardation assay of cts15-His6 with a 261-bp *Pvu*II fragment from the D3112 left end. Lanes 1–7, 5000 cpm radiolabeled fragment with 500 ng of purified cts15-His6 incubated with increasing amounts competitor DNA. Lane 8, 5000 cpm labeled fragment alone. The arrows in B indicate the two bands whose migration was inhibited upon incubation with cts15-His6. (C and D) Band retardation assays as in A and B except that 500 ng of purified Δcts15-His6 was used.

able phage D3112. Previous inspection of the D3112 left-end sequence (Autexier *et al.,* 1991) revealed the existence of a single large ORF commencing with a GTG codon. However, the start of the ORF was ambiguous due to the presence of three internal in-frame GTG codons that could also be used as the start codon for c repressor translation. We therefore mutated each of the four GTG codons to a GTA codon. Although this change preserves the incorporation of a valine and does not affect internal codons, the new GTA codon is unable to act as a start codon for translation of the protein (Neidhardt *et al.,* 1990). The mutagenized *c* ORFs were subcloned into the broad-host-range vector pTJS140 (Darzins and Casadaban, 1989a) and transformed into *P. aeruginosa* PAO1 cells. Lawns of these transformed cells were then tested with various dilutions of a D3112 phage lysate and incubated overnight to allow infection to occur (*in vivo* immunity assay). Plasmids with GTG-to-GTA mutations at either the first, third, or fourth GTG conferred on PAO1 immunity to D3112 superinfection at all dilutions of the D3112 phage lysate tested, as did plasmid pSWL12, which contains the unmutated *c*ts15 ORF (Table 1). In contrast, plasmid pKAS306, which has the second GTG converted to at GTA, was unable to confer immunity to D3112 superinfection, indicating that the second GTG is the probable start codon for the lysogenic c repressor. A closer look at the sequence directly upstream of the second GTG (Autexier *et al.,* 1991) reveals the presence of a 5/6 consensus Shine-Dalgarno ribosome recognition sequence (5′-GGAGG-3′) (Shine and Dalgarno, 1974). In addition the GeneMark.hmm program (Lukashin and Borodovsky, 1998) identifies the second GTG as the most likely translational start for the c repressor.

Having identified the proper start codon, we used a secondary structure prediction algorithm to identify six putative α-helices within the D3112 repressor (McClelland and Rumelhart, 1988; Kneller *et al.,* 1990). Based on the atomic structures of lambda repressor and Cro proteins (Branden and Tooze, 1991), we propose that the second and third putative α-helices constitute the DNA-binding, helix-turn-helix (H-T-H) motif. We also prepared a sequence alignment using BLAST (Altschul *et al.,* 1997) of D3112 repressor with its closest relative, the cI repressor from the λ-like *P. aeruginosa* bacteriophage D3

(Farinha *et al.,* 1994) (Fig. 1). The two proteins share 64.3% similarity (including 33.6% identity). The H-T-H motifs of the two proteins do not align well (data not shown), and the majority of the similarity appears to lie within the C-termini of these proteins. Farinha *et al.* (1994) predicted that the C-terminus of the D3 cI repressor contains the dimerization domain, whereas the putative H-T-H motif is located within the N-terminal region. A similar organization of motifs probably occurs within the D3112 repressor; five of the six putative α-helices occur in the N-terminal half of D3112 c.

Sequencing of the wild type D3112 c^+ gene and comparison with the D3112 *c*ts15 mutant (Autexier *et al.,* 1991) revealed a single AT→GC base pair mutation that results in an amino acid change from a glycine, in the wild-type repressor at position 6, to an aspartic acid, in the temperature-sensitive repressor (Fig. 1; putative α-helix 1). Interestingly, the change of a glycine to an aspartic acid leading to a temperature-sensitive protein function is found in at least five other proteins: the Mu c repressor (Vogel *et al.,* 1991), phage T4 lysozyme (Gray and Mathews, 1987), *Escherichia coli* ribosomal protein L24 (Nishi *et al.,* 1987), phage P22 tail spike endorhamnosidase (Yu and King, 1984), and the λ cI repressor (Nelson *et al.,* 1983; Groisman *et al.,* 1984). These temperature-sensitive changes occur either at the start of, or just before, a putative α-helix. In both D3112 c and Mu c repressors (Vogel *et al.,* 1991), the ts mutation occurs in the first putative α-helix of the repressor (Fig. 1).

To identify residues of the D3112 c repressor protein required for proper functioning, we used a random mutagenesis protocol. Mutagenesis of the complete repressor coding region, present in plasmid pSWL12 (Autexier *et al.,* 1991), was performed with the chemical mutagen hydroxylamine (HA). HA causes predominantly GC→AT (and sometimes AT→GC) transitions, mainly as a result of its reaction with cytosine (Freese *et al.,* 1961a,b; Tessman *et al.,* 1967). Of 100 mutated plasmids identified by a first screening, only 7 showed significant reduction in function after a second screening. The mutations in all of these plasmids were localized to a region corresponding to a putative H-T-H motif within the N-terminal region of the protein (Fig. 1, Table 2). Of the seven D3112 repressor mutants sequenced in this study, five were found to have a mutation leading to the nonconservative change Leu22Pro. From our secondary structure predictions, this mutation appears to lie in the first α-helix of our predicted H-T-H motif (Fig. 2). Leucine is an aliphatic, hydrophobic amino acid, commonly found in regions of α-helices that do not have contact with the aqueous surroundings (Branden and Tooze, 1991). In contrast, proline is an imino acid and a known α-helix disrupter with a tendency to introduce sharp kinks in protein secondary structure. The Leu22Pro mutation may disrupt the first α-helix in the putative H-T-H motif such that the repressor can no longer fold correctly or bind DNA. The two other mutations that were found led to the changes Leu36Pro and Leu37Phe. Both residues localize to the second α-helix of the putative H-T-H motif. In other DNA-binding proteins, such as λ cI, the second α-helix of the H-T-H motif is involved in DNA recognition (Pabo and Lewis, 1982). Therefore, if this second α-helix is the c repressor DNA recognition α-helix, disruption would clearly abolish specific DNA binding by the repressor protein and result in the loss of activity of the protein. The Leu36Pro mutation would likely disrupt this third α-helix. However, the Leu37Phe change is not as obviously disruptive to protein structure, because both are aliphatic hydrophobic amino acids. One difference between the two, however, is that although leucine is a branched chain amino acid, phenylalanine contains a bulky aromatic ring. This difference may somehow destabilize protein function sufficiently to abrogate function. In conclusion, the observation that HA mutations that abolish repressor function map to the predicted H-T-H motif supports our prediction of secondary structure.

To further characterize the D3112 *c* repressor, we localized the promoter, P*c*. Fragments spanning the upstream regions of the *c* ORF were subcloned into the broad-host-range promoterless *lacZ* vector pDN19lacΩ (Totten and Lory, 1990) and used to transform the wild-type *P. aeruginosa* strain PAO1. The β-galactosidase assays (Fig. 2) identified the fragment spanning bp 1099–838 (a *Pvu*II fragment, pKAS232) as the smallest fragment having promoter activity. This indicates that the promoter, P*c*, resides within these 261 bp.

Primer extension analysis was used to determine the transcriptional start site of the *c* gene and to precisely map the position of P*c* (Fig. 4). A single detectable transcriptional start site was found to be located 154 bp upstream from the second GTG at position 940 and was centered 8 bp downstream of a putative σ^{70} promoter sequence [−10 (AACAGT)/−35 (CTGATT)] (Hawley and McClure, 1983). This same promoter region was also identified using the Neural Network Promoter Prediction algorithm (score: 0.98; Reese, 1994; Reese and Eeckman, 1995; Reese *et al.,* 1996). Finally, Northern blotting indicated that the *c* repressor mRNA is 900 nt long (Fig. 3), indicating that D3112 *c* is monocistronic as seen in the two coliphages Mu and D108 (Krause *et al.,* 1983; Levin and DuBow, 1989).

To overexpress and purify the D3112 repressor, the *c* gene, beginning with the second and third GTG codons, were amplified by PCR and cloned into the pET29-b expression vector. The purified D3112 cts15-His6 repressor protein (Fig. 5) bound specifically to a fragment located directly upstream of the *c* ORF (Fig. 6B), but the Δcts15-His6 protein (expressed from the third GTG) did not (Fig. 6D). D3112 cts15-His6 bound to the specific fragment produces two distinct bands on binding (Fig. 6B, lanes 3–7). The formation of these two bands suggests that there may be two operators located within this

fragment to which repressor binds, a result also observed with the c repressor from the transposable coliphage D108 (Kukolj and DuBow, 1991). Because both Mu and D108 repressors have been shown to bind specifically to operators located in positions analogous to this fragment, these data suggest that putative operators for D3112 repressor exist in this region.

Binding of cts15-His6, but not the Δcts15-His6 protein, to the specific fragment provides an additional line of evidence to support our assignment of the second GTG as the start codon. Furthermore, the predicted H-T-H (amino acids 19–43) would not be present within Δcts15-His6 because the location of the third GTG is at position 661 (amino acid 46 of the second GTG codon initiated protein). Because Δcts15-His6 failed to demonstrate specific binding activity to a fragment found directly upstream of the *c* ORF, this suggests that the DNA-binding domain is lacking from this protein and provides further support to our localization of the H-T-H motif.

The D3112 c repressor region has been shown to have an organization similar to that seen in the two coliphages Mu and D108. However, the c protein exhibits higher similarity to the λ-related family of cI repressor proteins. The location of the promoter, P*c*, in a position analogous to that seen in Mu and D108, and specific binding to this region, suggests an evolutionary relatedness to the two coliphages. Moreover, in Mu and D108, binding of the repressor to its operators is positioned to ensure the obstruction of transcription from the early promoter P*e*, and subsequent repression of transcription of the *ner* gene during lysogeny. D3112 has not yet been shown to possess a *ner* homolog in the analogous position (Bidnenko *et al.,* 1996; Krylov *et al.,* 1980a,b; Yanenko *et al.,* 1983, 1988). The localization of the other components of early gene regulation will be important in the understanding of D3112 c repressor function in the lytic-lysogenic switch of this *Pseudomonas* transposable bacteriophage.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and culture conditions

The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 3. All cultures were grown at 37°C in Luria Bertani broth and agar (Sambrook *et al.,* 1989) except for PAS429 and strains containing plasmids expressing the temperature-sensitive c repressor (cts), which were grown at 32°C. When necessary, antibiotics were added at the following concentrations: for *P. aeruginosa*, pipercillin (100 μg/ml), streptomycin (300 μg/ml), and tetracycline (200 μg/ml), and for *E. coli*, ampicillin (50 μg/ml), kanamycin (50 μg/ml), streptomycin (100 μg/ml), and tetracycline (10 μg/ml).

Enzymes and chemicals

All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Superscript II Reverse Transcriptase were purchased from either Amersham-Pharmacia (Baie d'Urfe, Quebec, Canada) or GIBCO BRL (Burlington, Ontario, Canada). *Taq* DNA polymerase was purchased from Promega (Madison, WI). The Sequenase Version 2.0 Kit was purchased from Amersham-Pharmacia, and the Isotherm Sequencing Kit was purchased from Epicenter Technologies Inc. (Madison, WI). All other chemicals were purchased from either Fisher (Nepean, Ontario, Canada) or Sigma Chemical Co. (St. Louis, MO).

Bacteriophage lysates and *in vivo* immunity assay.

Bacteriophage lysates were prepared using strain PAS429 grown at 32°C until an A_{550} value of 0.6, shifted to 42°C, and incubated until the A_{550} was less than 0.1. The phage lysate was titered on TCMG plates (Schumm *et al.,* 1980) as previously described (Ljundquist and Bukhari, 1977). The *in vivo* immunity assay was performed as previously described (Levin and DuBow, 1989).

Site-directed mutagenesis

Site-specific mutagenesis was performed with the U.SE. Mutagenesis Kit (Pharmacia Biotech) using the *Sca*I–*Mlu*I U.S.E selection primer (Pharmacia Biotech) for the initial screening procedure, according to the manufacturer's instructions. Primers KS15 (5′-GTCTGATTTTACGATAAAAAAGCCG-3′), KS16 (5′-GCAGCCTAGCTACCTCCGCG-3′), KS17 (5′-CCAATCCAGACTTACACGACCAGCC-3′), and KS18 (5′-GGCTTCGCAGCTACCTCTTCCCC-3′) were used to specifically mutagenize the first, second, third, and fourth GTG codons to GTA codons (underlined) in the *c* repressor gene. The primers were purchased from GIBCO BRL.

PCR amplification

PCRs were performed in a DNA Thermal Cycler (MJ Research, Inc.). For PCR amplification of the various *c* ORFs, the reactions were performed in 50-μl volumes. Each reaction mixture contained 50 ng of DNA template, 2 U of *Taq* DNA polymerase, 4 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate mix, and 0.2 μM primers (see later). Thirty cycles were performed for each reaction. Each cycle consisted of incubations for 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C. The primers used for the PCRs were purchased from GIBCO BRL. Restriction enzyme sites were added at the 5′ ends of the primers (shown below in bold) to facilitate subsequent cloning of the PCR products. Additional nucleotides were added 5′ to the restriction enzyme sites to ensure efficient cleavage. The following primers were used in the PCRs: primers KS6 (5′-CCCC**CATATG**-GCTAGGCTGC-3′) and KS25 (5′-CCCC**CTCGAG**AACCA

TABLE 3

Bacterial Strains, Phages, and Plasmids Used in This Study

Strain/phage/plasmid	Relevant characteristics	Reference/origin
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type, prototroph	Holloway, 1969
PAS429	PAO1 *ami*::D3112*c*ts15	J. Shapiro
Escherichia coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)	Studier and Moffatt, 1986
DH5α	*supE*44 Δ*U*169 (ϕ80 *lacZ*Δ*M*15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan, 1983
Phages		
D3112 *c*ts15	*c*ts, temperature-sensitive repressor (38kb)	J. Shapiro
MK1307	ssDNA helper phage	Vieira and Messing, 1987
Plasmids		
pBR322	Ap^R, Tc^R, cloning vector	Bolivar *et al.,* 1977
pUC119	Ap^R; cloning vector	Vieira and Messing, 1987
pET29-b	Km^R; C-terminal 6XHIS expression vector	Novagen, Inc.
pDN19lacΩ	Str^R/Spc^R, Tc^R; broad host range, promoterless *lacZ* vector	Totten and Lory, 1990
pTJS140	Ap^R; rep$_{RK2}$ *oriT*$_{RK2}$ rep$_{pMB1}$ *lac′IPOZ*	Darzins and Casadaban, 1989a
pB28$_L$	Left-end (backfilled-*Hin*dIII) fragment of D3112 *c*ts15 into the *Sma*I-*Hin*dIII site of pUC119	Autexier *et al.,* 1991
pSWL12	*Nru*I–*Eco*RI fragment from pB28$_L$ into the *Sma*I–*Eco*RI site of pTJS140	Autexier *et al.,* 1991
pOF4	Left-end (backfilled-*Hin*dIII) fragment of D3112c$^+$ into the *Sma*I–*Hin*dIII site of pUC119	This study
pOF6	*Nru*I–*Eco*RI fragment from pOF4 into the *Sma*I–*Eco*RI site of pTJS140	This study
pKAS220	bp 1174–bp 366 fragment of D3112*C*ts cloned into the *Eco*RI–*Bam*HI site of pDN19lacΩ	This study
pKAS224	bp 1172–bp 518 fragment of D3112*C*ts cloned into the *Eco*RI–*Bam*HI site of pDN19lacΩ	This study
pKAS228	bp 1172–bp 772 fragment of D3112*C*ts cloned into the *Eco*RI–*Bam*HI site of pDN19lacΩ	This study
PKAS230	bp 838–bp 1099 *Pvu*II fragment of D3112*c*ts cloned into the *Sma*I site of pUC119	This study
pKAS305	*Nru*I–*Eco*RI fragment from pB28$_L$ (1st GTG to GTA) into the *Sma*I–*Eco*RI site of pTJS140	This study
pKAS306	*Nru*I–*Eco*RI fragment from pB28$_L$ (2nd GTG to GTA) into the *Sma*I–*Eco*RI site of pTJS140	This study
pKAS307	*Nru*I–*Eco*RI fragment from pB28$_L$ (3rd GTG to GTA) into the *Sma*I–*Eco*RI site of pTJS140	This study
pKAS308	*Nru*I–*Eco*RI fragment from pB28$_L$ (4th GTG to GTA) into the *Sma*I–*Eco*RI site of pTJS140	This study
pOF7	pSWL12/L22P	This study
pOF8	pSWL12/L36P	This study
pOF14	pSWL12/L37F	This study

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Spc^R, spectinomycin resistance; Str^R, streptomycin resistance, Tc^R, tetracycline resistance.

TCCAGCGGC-3′) were used for the PCR amplification of the *c* ORF from the second GTG start codon, and KS12 (5′-CCCCCC**CATATG**AGTCGGATTGGC-3′) and KS25 were used for the PCR amplification of the *c* ORF from the third GTG. In each case, one of the primers (KS6 or KS12) modifies the original GTG start codon such that there is an ATG within the PCR product.

HA mutagenesis of pSWL12

CsCl-purified pSWL12 plasmid DNA was mutagenized using HA as previously described (Miller, 1972) for 24 h at 37°C. After mutagenesis, the DNA was dialyzed in 2 liters of T.E. buffer (Sambrook *et al.,* 1989) at 4°C for 24 h with two changes of T.E. buffer and then ethanol precipitated as previously described (Miller, 1972).

DNA sequencing

Single-stranded DNA required for sequencing reactions was obtained by superinfection with phage M13K07 (Vieira and Messing, 1987) or by denaturing double-stranded DNA as described (Lim and Pene, 1988). DNA sequencing was performed using either the Sequenase

version 2.0 Kit or the Isotherm Sequencing Kit according to the manufacturer's instructions. Sequencing reactions were subjected to electrophoresis on either 5% polyacrylamide or 5% Long Ranger (JT Baker, Phillipsburg, NJ) sequencing gels as previously described (Maxam and Gilbert, 1980) and visualized by autoradiography after exposure to Kodak XAR-5 film for 24–72 h at −70°C under DuPont Cronex intensifying screens.

Plasmid constructions

Plasmids used for the *in vivo* immunity assay were constructed in the following manner: Plasmid $pB28_L$, containing the leftmost 1.8 kbp of the D3112*c*ts15 genome in pUC119 (Autexier *et al.,* 1991), was used as the target plasmid for the site-specific mutagenesis. In four separate reactions, each of the four potential GTG start codons was replaced with the non-f-methionine codon GTA, generating plasmids pKAS301, pKAS302, pKAS303, and pKAS304. The plasmids were sequenced to confirm that no other mutations had been incorporated into the mutagenesis product. The plasmids were then hydrolyzed with *Nru*I–*Eco*RI, and the resulting fragments were subcloned into the *Sma*I–*Eco*RI sites of the broad-host-range plasmid pTJS140, producing plasmids pKAS305, pKAS306, pKAS307, and pKAS308 (Table 2.1).

Cloning of the left end of D3112 c^+ phage DNA for sequencing and functional studies was accomplished by first ensuring that the left end of the D3112 c^+ was blunt with T4 DNA polymerase (Sambrook *et al.,* 1989). The genome was then hydrolyzed with *Hin*dIII, and a 1.8-kbp DNA fragment was isolated from a 0.7% agarose gel using Gene Clean (Bio 101, Vista, CA) according to the manufacturer's instructions. This fragment was then ligated, using T4 DNA ligase, to the expression vector pUC119 previously hydrolyzed in its polylinker with *Sma*I and *Hin*dIII, resulting in the plasmid pOF4. Cloning of the D3112 c^+ repressor gene into a broad-host-range vector was accomplished by hydrolyzing pOF4 with *Nru*I and *Eco*RI and isolating a 1.1-kbp fragment (as described earlier). This fragment was then ligated, using T4 DNA ligase, to vector pTJS140 that had been previously cleaved in its polylinker at the *Sma*I and *Eco*RI sites generating plasmid pOF6.

From the mutagenesis experiments, HA-treated pSWL12 plasmids (see earlier) that lost the ability to provide immunity to superinfection *in vivo* were digested with *Pst*I and *Eco*RI, and a 1.1-kbp fragment was isolated (as described above). This fragment was then ligated to a nonmutagenized pTJS140 vector previously digested with *Pst*I and *Eco*RI. The resultant plasmids (named pOF7, pOF8, pOF9, pOF10, pOF12, pOF14, and pOF15) were again screened for loss of immunity function. For these mutant plasmids, the *Pst*I–*Eco*RI fragment was also ligated into the vector pUC119, previously cleaved in its polylinker with *Pst*I–*Eco*RI, for sequencing (pOF107, pOF108, pOF109, pOF110, pOF112, pOF114, and pOF115).

Plasmids for the promoter determination via β-galactosidase promoter-probe fusions were constructed in the following manner: various fragments from the D3112*c*ts15 genome (see Fig. 2) were made blunt using T4 DNA polymerase (if required), subcloned into the *Sma*I site of pUC119 and the orientation determined by restriction enzyme hydrolyses. Plasmids containing the proper orientation were hydrolyzed with *Eco*RI and *Bam*HI and the resulting fragments isolated and subcloned into the *Eco*RI–*Bam*HI sites of pDN19lacΩ (Totten and Lory, 1990). The resulting plasmids are listed in Table 3.

Plasmids for the overexpression of D3112 *c*ts15, using either the second or third GTG as the start codon, were constructed in the following manner: the PCR products from each ORF were hydrolyzed with *Nde*I–*Xho*I and ligated to the *Nde*I–*Xho*I site of pET29-b. Once constructed, the plasmids were sequenced using the T7 promoter primer and the T7 terminator primer (Novagen, Inc., Madison, WI) to ensure there were no alterations arising from the PCR reactions. The resulting plasmids were called pKAS210 and pKAS211, respectively (Table 3).

Transformations and electroporations

Transformations of *E. coli* were performed using an $RbCl_2$ method (Hanahan, 1983). Electroporation of *P. aeruginosa* strains was performed according to the method of Farinha and Kropinski (1990) using 15% glycerol, 1 mM MOPS as the buffer. The DNA used for the transformations and electroporations was prepared using either an alkaline-lysis method (Sambrook *et al.,* 1989) or Qiagen quick-spin columns (Qiagen, Mississauga, Ontario, Canada).

β-Galactosidase assays

Expression of the *lacZ* gene, under the control of the putative P*c* promoter region, was measured using β-galactosidase assays as described by Miller (1972).

Northern blot hybridization

Total cellular RNA was prepared from *P. aeruginosa* strains PAO1, PAS429 (grown at 32°C), and $PAS429^{IND}$ (PAS429 grown at 32°C to $A_{550} = 0.7$ and then induced for 8 min at 42°C) by an adaptation of the CsCl purification procedure (Glisin *et al.,* 1974), as described by Deretic *et al.* (1987). RNA, 10 μg from each strain, was subjected to electrophoresis on a 1.2% agarose–formaldehyde gel (Ausubel *et al.,* 1989), transferred, and fixed to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as described previously (Cai and DuBow, 1996). The 0.4- to 9.5-kb RNA ladder (GIBCO BRL) was subjected to electrophoresis in parallel with the above samples and visualized by ethidium bromide staining before transfer

to the nitrocellulose membrane. A 0.6-kb *Nde*I–*Xho*I fragment from pKAS210, containing the entire D3112 *c* gene, was used to detect D3112 *c* mRNA (Table 3). The DNA probe was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, 1 Ci = 37 Gbq; Amersham-Pharmacia) using the random priming method (Sambrook *et al.,* 1989) and random hexanucleotide primers (GIBCO BRL). Hybridization was carried out as previously described (Mahmoudi and Lin, 1989). Unhybridized probe was removed by washing the filters twice at 68°C in 2× SSC, 1% SDS. The blots were air dried and exposed to a Molecular Dynamics Storage Phosphor Screen and visualized using a PhosphorImager SF and Image Quant Software Program (Molecular Dynamics, Sunnyvale, CA).

Transcriptional start site determination

The transcriptional start site of the *c* gene was determined by primer extension. Primer KS21 (5′-GCGTCGAAGTCGGAACAGC-3′), which complements the noncoding strand near the 5′ end of the *c* ORF, was end-labeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham-Pharmacia) using T4 polynucleotide kinase according to the manufacturer's instructions (GIBCO BRL). The labeled primer was purified using the Nucleotide Removal Kit (Qiagen Inc., Mississauga, Ontario, Canada), and the resulting labeled primer was used for the reverse transcription (RT) reactions. The total RNA template for the primer extension reaction was prepared as described above and was pretreated with DNase I (Boehringer-Mannheim) before the RT reaction. Superscript II reverse transcriptase was used for the RT reactions under the conditions recommended by the manufacturer (GIBCO BRL). The DNA template for the sequencing reaction was $pB28_L$ and the primer was KS21. The products of the sequencing and RT reactions were subjected to electrophoresis on a 5% polyacrylamide sequencing gel and visualized using autoradiography as described earlier.

Overexpression and purification of D3112 *c*ts15

E. coli BL21(DE3) cells were transformed with plasmid pKAS210 or pKAS211 or the vector control plasmid pET29-b. Bacterial cultures were grown to an A_{550} value of 0.4, and expression of T7 RNA polymerase, required to transcribe the T7 promoter in the plasmids, was induced in the host strain by the addition of isopropyl thiogalactopyranoside (IPTG) to a final concentration of 1 mM (Studier and Moffatt, 1986). The cultures were grown for an additional 3 h and harvested by centrifugation for 20 min at 4000 *g* at 4°C. Purification of the two proteins were performed under native conditions using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions with the following modifications: the lysis buffer contained 5 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF). Two different wash buffers were used containing 1 mM PMSF and either 20 or 50 mM imidazole, respectively; the elution buffer contained 250 mM imidazole and 1 mM PMSF. Samples of the crude extracts, washes, and elutions were subjected to electrophoresis on a 20% SDS–polyacrylamide gel (Laemmli, 1970).

Operator binding assay

Binding of the D3112 repressor to DNA was assayed using the gel electrophoretic shift method (Fried and Crothers, 1981; Garner and Revzin, 1981). The buffer used in the binding reactions contained 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 50 mM NaCl, 100 μg/ml BSA, and specified amounts of sonicated calf thymus DNA. Protein–DNA binding reactions were performed and then subjected to electrophoresis on 5% polyacrylamide gels as described in Kukolj and DuBow (1991).

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